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THE USE OF MICE IN TESTS OF IMMUNITY AGAINST YELLOW FEVER*

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The Need for a Test of Immunity against Yellow Fever

The study of the epidemiology of yellow fever would be greatly facilitated by some simple immunity test which could be applied to human beings on a large scale. With such a test it would be possible to determine whether yellow fever has been present in a region within the life of the present generation and to estimate the time and extent of the infection. By testing an adequate number of persons in selected places, it should be possible to map large areas with regard to the endemicity, epidemicity, or absence of yellow fever, and to decide where control measures are necessary. The test would be especially valuable where the disease is present but is seldom or never recognized, and also where there is confusion of yellow fever with other diseases, particularly leptospiral jaundice. Such a test is needed also for determining whether persons vaccinated against yellow fever by any of the proposed methods have in fact been immunized. Just at present there is a pressing demand for immunity tests in connection with yellow fever surveys of West Africa, whence aviation and other improved means of travel threaten to carry yellow fever past the little understood natural barriers to large susceptible populations in East Africa and the Orient. Numerous immunity tests will be needed also for delimiting the endemic areas of Brazil.

A very simple method of ascertaining whether a person is immune to yellow fever would be by a skin test, if a suitable antigen could be

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found, but Frobisher (1) was unable to produce significant skin reactions in immune monkeys by the intradermal injection of any of several antigens. The methods of testing sera for their power to fix complement in the presence of yellow fever antigens, as developed by Frobisher (2) and Gordon Davis (3), seem to give specific results, but fixation does not take place as a rule in the tests of the sera of animals which have been immunized by vaccination without the production of fever, or in the sera of some of the highly immune persons who have recently had yellow fever (Hudson (4)). While the method gives promise, it will not be ready for wide application, except as a supplementary test, until its limitations and methods of interpretation have been more exactly determined. The protection test in *Macacus rhesus* monkeys, on the other hand, has proved reliable and highly useful, but it cannot be applied on a scale large enough for present needs because too great a number of monkeys would be required. By testing human sera for their power to protect monkeys against yellow fever virus, Beeuwkes, Bauer, and Mahaffy (5) were able to determine the endemicity of yellow fever in parts of West Africa where cases were seldom recognized, and to show that certain other regions were free of the infection. By this same test the identity of the recent isolated outbreak of yellow fever in the interior of Colombia, described by Peña Chavarría, Serpa, and Bevier (6), was confirmed in the laboratory in New York by Hudson and Kitchen (7).

The protection test has many advantages and would meet the needs of the investigators in the field if a larger number of sera could be examined than is possible when monkeys are used. Any one of the potent African strains of yellow fever virus could be used in testing sera from any part of the world, for the serological identity of the African and American strains has been amply demonstrated by cross-immunity tests (Sawyer, Kitchen, Frobisher, and Lloyd (8)) and by the growing experience with protection tests in which sera from many parts of the Americas have protected against African strains of virus. The protection test can, as a rule, be depended upon to reveal immunity resulting from attacks of yellow fever no matter how long past. For example, specimens of serum from six persons who were ill during the epidemic in Norfolk, Virginia, in 1855 were obtained 75 years later, and five of the six sera protected monkeys against African yellow fever

virus (Sawyer (9)). Protection tests of the sera of a sufficient number of persons classified by location and age group would permit the reconstruction of at least the recent yellow fever history of a region and would thus help in solving the present problem of control.

The Susceptibility of the Mouse to Yellow Fever

When the susceptibility of the white mouse to the virus of yellow fever was announced in 1930 by Theiler (10, 11), it seemed that the laboratory animal needed for replacing the monkey in the protection test had been found. Working with the French strain of yellow fever virus from Africa, he discovered that adult mice could be infected by intracerebral inoculation but not ordinarily by subcutaneous or intraperitoneal injections.

Theiler found that the virus was highly neurotropic in mice and was abundant in the brain tissue after the disease had developed, although absent from the blood or liver. There was a heightening of the virulence for mice with successive passages in these animals, until a fixed strain for mice was produced which caused the death of all the animals inoculated intracerebrally in approximately 5 days after injection. Theiler showed that experimental yellow fever in mice took the form of an encephalitis with characteristic changes in the nuclei of the ganglion cells. Outside the nervous system the only lesion found which might be characteristic of yellow fever was hemorrhage into the stomach and intestines, but this occurred only in some of the infected animals. He showed also that the sera of immune monkeys or persons had the power to prevent or defer the disease if mixed with the virus before the intracerebral injection. Variation of the length of time that the virus and serum were in contact before injection seemed to make no difference in the degree of protection. Injections of large amounts of immune serum intraperitoneally seldom protected against virus inoculated intracerebrally.

The Protection Test by Intracerebral Inoculation of Mice

In a later publication Theiler (12) has presented experiments with a method for testing sera for their protective power against the fixed virus for mice. He added each immune serum to an equal volume of a suspension of the virus, and inoculated mice intracerebrally with the mixture, using six or more mice in each test. Control groups of mice received normal serum in the place of the immune serum. Nearly all the control animals died from 5 to 9 days after inoculation. Of the mice which had received immune serum, usually a few died like the control animals, a few more died from the 10th to the 16th day, and several survived. Even when the virus suspension contained only 1 per cent of virus and had been centrifuged to remove all particles, a considerable proportion of the animals which received immune serum and virus died within the period of observation. The test obviously

permitted differentiation between immune and non-immune sera, but the irregularity of the results would make it necessary to use an excessive number of animals to reach valid conclusions in testing large numbers of specimens. If this test can be made more highly reliable by quantitative adjustments, it will have the advantage over the modified test which we shall describe of requiring only very small quantities of serum and virus.

Establishment of New Strains of Yellow Fever Virus in Mice

In our study, the susceptibility of the white mouse to yellow fever virus when inoculated intracerebrally was confirmed.

The Asibi strain of yellow fever virus of African source was established in mice by our associate in the laboratory, S. F. Kitchen, and we likewise established the S. R. strain from Brazil and the P. A. L. strain. Theiler kindly let us have the French strain in its 95th passage in mice, and most of our work was done with this strain, which has now reached its 141st passage. The Asibi strain has been carried through 51 passages, the S.R. through 21, and the P.A.L. through 15. These new strains in mice have gradually become more like the fixed French strain, but only the Asibi strain has reached a point at which it may be considered as fixed. In Table I are shown the intervals in days between the intracerebral inoculation of mice with each of these strains and the time when the animals were found dead or were killed when definitely ill. The periods shown give a close approximation to the incubation periods, as the time interval between the observance of definite symptoms of weakness or partial paralysis and the death of the animal is usually short, often only a few hours but not infrequently 1 day. Many of the mice inoculated with the French strain in the latest series in the table were killed so that their brains could be used for inoculation. If they had not been killed, a few of them would doubtless have died during the following night and their deaths would have been recorded as occurring 1 day later. The differences shown in the table between the results with the earlier and the later passages of the French and Asibi strains of virus are of doubtful significance. The virus was already fixed in the earliest passages available for the comparison. It is obvious, however, that both these fixed strains produce death in a shorter time than do the two strains which have been passed through mice only a small number of times. The mortality is not shown in the table, as nearly all the mice died that were used in the experiments on which the table is based.

Observations made before Nov., 1930, are excluded from Table I, as many of our earlier experiments were made with a strain of mice of low susceptibility to yellow fever virus, and the results were therefore not comparable with those in the table.

With Kitchen we have fully confirmed Theiler's observations (10) that yellow fever virus, after a number of passages in mice, will still

produce experimental yellow fever with typical lesions in *rhesus* monkeys, but that the virus finally loses its power to produce fatal attacks in monkeys after many passages in mice. We shall reserve details of these observations for a later paper by Kitchen and ourselves dealing with vaccination against yellow fever. The pathology of experimental yellow fever in mice is being studied by Kitchen and will be described by him in a later publication.

Protection Test by Intraperitoneal Inoculation of Mice

In applying the protection test by intracerebral inoculation, we obtained results similar to those of Theiler, and came to the conclusion

TABLE I

Effect of Numerous Passages of Yellow Fever Virus of Several Strains on the Time Interval between Intracerebral Inoculation and Death

Strain of yellow fever virus	No. of passages in mice	No. of animals dying, or killed when ill, after each of the following intervals in days after inoculation								
		4	5	6	7	8	9	10	11	12 to 14
French	Earlier: 105, 106, 107	3	35	21						
	Latest: 138, 139, 140	133	276	7	2					
Asibi	Earlier: 32, 33, 34	5	17	4	4					
	Latest: 48, 50, 51	3	8	14	4					
S. R.	Early: 3, 4,				6	5	4	1	1	
	Latest: 19, 20, 21		4	11	6	2				
P. A. L.	Early: 5, 6, 7	1	4	13	12	10				
	Latest: 13, 14, 15		3	7	7	5				

that we could not expect the small amount of immune serum injected intracerebrally to protect completely and regularly against fixed yellow fever virus placed in direct contact with injured brain cells. We therefore sought some method by which more serum could be injected and the virus could be brought less directly and more slowly to the brain.

It seemed probable that massive intraperitoneal injections of the virus in brain tissue would cause the introduced virus to circulate in the blood for several days, for Sawyer and Frobisher (13) had shown

that yellow fever virus could be recovered for 2 or more days from the heart blood of several resistant animals inoculated in this way (guinea pig, ferret, bullfrog). This circulation of virus was evidently not necessarily related to any infection either inapparent or obvious which might develop later, because it occurred in the entirely insusceptible bullfrog. To enable the circulating virus to attack the brain of the adult mouse it might be necessary to cause some mild injury of the brain tissues, because simple intraperitoneal inoculation alone does not produce encephalitis in the adult animals. If infection could be brought about in this way, it should be possible to test sera for protective power by injecting them intraperitoneally in adequate amounts together with the virus and at the same time producing a mild injury to the brain.

There were precedents for the application of the principles involved in the method proposed. Flexner and Amoss (14) reported in 1917 that "normal monkey or horse serum, isotonic salt solution, and Ringer's and Locke's solutions, when injected into the meninges, promote infection with the virus of poliomyelitis introduced into the blood, the nose, or the subcutaneous tissues." Zwick, Seifried, and Witte (15) found out that the cutaneous inoculation of rabbits with Borna virus succeeds only in exceptional instances, but that infection is easily brought about by such inoculation if at the same time an injury is produced in the central nervous system by the injection of non-specific fluids (normal rabbit serum, normal horse serum, or isotonic sodium chloride solution).

The Agent for Localizing the Virus in the Brain

In preliminary trials many fluids were injected intracerebrally into mice and compared for their effect in localizing yellow fever virus in the brain.

The virus was injected intraperitoneally, as 0.2 cc. of a 10 per cent suspension of infected mouse brain, immediately after the anesthetized mouse had been given the intracerebral injection. The substances tested as the intracerebral inoculum were distilled water, 0.9 per cent and 3 per cent sodium chloride solutions, normal human and normal horse sera, solutions of magnesium chloride, magnesium sulfate, potassium alum, and manganese chloride, 10 per cent extract of mouse testicle in 0.9 per cent sodium chloride solution, and solutions of corn-starch, tapioca, and wheat flour. Simple needling of one or both sides of the brain was tried also. Some of the salts were too toxic in the strengths of the solutions used and were abandoned. Others of the solutions seemed to have uncertain or inadequate effects.

Satisfactory results were obtained with each of the three solutions of starch in either distilled water or 0.9 per cent sodium chloride solution. The 0.5 per cent magnesium chloride solution seemed almost equally effective and was used in a considerable number of the earlier tests. Our final choice of the fluid for injection in routine tests was a 2 per cent solution of starch in 0.9 per cent sodium chloride solution injected intracerebrally in the amount of 0.03 cc., and this solution has been used in all the more recent tests.

At the time of these early comparative tests we were using strains of mice of low and varying susceptibility and, as a result, an accurate comparison was impossible. Working more recently with highly susceptible mice, we repeated many of these experiments and found that most of the mice succumbed to yellow fever encephalitis regardless of which substance was injected, and even when the conditions were made less favorable by a reduction in the amount of virus inoculated intraperitoneally. Simply inserting the hypodermic needle into the brain often proved sufficient to establish the infection. The results of these recent tests are given in Table II. While these tests would seem to allow considerable latitude in the choice of the intracerebral inoculum, we have decided to continue using the 2 per cent starch in isotonic sodium chloride solution. It seemed to us probable that the slower absorption of the starch and the presumably more definite injury to the tissues would make it more reliable than simple needling or the injection of isotonic salt solution alone. In any event the starch solution is proving effective in our experience with the protection tests in mice.

The Intraperitoneal Inoculum

In the production of experimental yellow fever in mice by the simultaneous injection of virus into the peritoneal cavity and a mildly injurious substance into the brain, we found that a considerable proportion of the animals escaped infection unless the amount of virus injected was large.

In one comparative test the brain injury was produced by the injection of starch solution and the intraperitoneal injection of virus consisted of a 10 per cent suspension of the brain of an infected mouse in isotonic salt solution. An equal volume of normal human serum was injected with the virus. When the amount

TABLE II

Comparative Tests of Mechanical Injury and the Injection of Various Fluids for Their Power to Localize Yellow Fever Virus in the Brain of the Mouse

Fluid injected into brain to localize the virus (0.03 cc.)	Percentage of infected mouse brain in intraperitoneal inoculum (0.2 cc.)*	No. of mice inoculated and alive 4 days later	No. dying from 5 to 10 days after inoculation	Per cent dying within this period
No fluid (only insertion of needle)	20	12	10	83
	5	12	7	58
	1	11	8	73
0.9 per cent sodium chloride	20	12	10	83
	5	12	11	92
	1	12	6	50
3 per cent sodium chloride	20	6	3	50
	5	11	8	73
	1	11	4	36
0.5 per cent magnesium chloride†	20	12	10	83
	5	11	11	100
	1	10	6	60
Normal horse serum	20	12	10	83
	5	11	6	55
	1	9	6	67
20 per cent extract of mouse testicle†	20	12	10	83
	5	11	10	91
	1	10	5	50
2 per cent starch solution†	20	12	10	83
	5	12	9	75
	1	12	7	58
Combined results with all of above injurious agents	20	78	63	81
	5	80	62	78
	1	75	42	56

* Virus: French strain 139th and 140th passages in mice. Tests with the 20 per cent suspension were made together as one experiment, and the other tests comprised a second experiment. Each mouse received 0.4 cc. of normal human serum in the first experiment and 0.4 cc. of a 10 per cent solution of normal human serum in 0.9 per cent sodium chloride solution in the second.

† Dissolved or suspended in 0.9 per cent sodium chloride solution.

of the brain suspension injected was 0.5 cc., all of ten mice inoculated died, but when the amount was 0.1 cc., only three of five mice died. In another experiment the concentration of the virus suspension was varied while the volume was kept constant at 0.2 cc. Normal human serum (0.4 cc.) was injected with the virus into each animal. The intracerebral injection consisted of 0.03 cc. of 2 per cent starch.

As is shown in Table III, the animals receiving virus in brain suspensions of a concentration of 10 per cent, or over, all succumbed, while a considerable proportion of those receiving suspensions of 1 per cent or less survived. Likewise in the experiments reported in

TABLE III

Variation of the Mortality Rate of Mice with the Amount of Virus in the Intraperitoneal Inoculum

Mouse brain in virus suspension*	No. of mice inoculated and alive 4 days later	No. of these mice dying 5 to 10 days after inoculation	Mice dying
per cent			*per cent*
40	4	4	100
20	5	5	100
10	11	11	100
1	5	3	60
0.1	8	1	13
0.01	8	1	13

* Virus: French strain, 111th passage in mice. Volume of the virus suspension injected intraperitoneally, 0.2 cc. With it was mixed 0.4 cc. of normal human serum. Simultaneous intracerebral inoculum: 0.03 cc. of 2 per cent starch solution.

Table II the number of fatal infections was much smaller when the intraperitoneal inoculum was a 1 per cent suspension of virus than when it was a 5 per cent suspension of the same virus.

Evidently the chance of producing encephalitis in all the mice of a group inoculated by the method used is greater when the larger quantities of virus are injected. There are practical difficulties, however, without compensatory advantages in using very large amounts.

The observations in Table II show that the use of even as much as 0.2 cc. of a 20 per cent suspension of brain virus may allow some of the animals to survive, and the experience of the laboratory, in testing several hundred sera, was that

this amount of virus would often permit one mouse to survive, and sometimes two, in a group of six control animals receiving the virus with normal human serum.

To secure the necessary amount of 20 per cent virus suspension for the tests, approximately as many mouse brains were required as there were specimens to be tested, and this made necessary the inoculation of many animals as a source of virus. As the substitution of 10 per cent virus for 20 per cent made only a small difference in the accuracy of the test, when highly susceptible animals were used, and resulted in a great saving of animals, we have recently adopted 0.2 cc. of 10 per cent mouse brain virus as the virus component of the intraperitoneal inoculum, and have paid special attention to the securing of suitable mice.

Isotonic sodium chloride is the fluid in which the brain was at first suspended in the preparation of the virus for inoculation, and it is satisfactory if the virus is freshly prepared just before it is mixed with the undiluted sera to be tested. In the light of the experience of Bauer and Mahaffy (16), who found that the addition of 10 per cent of normal monkey serum to diluted virus suspensions helped preserve the virus, we prefer to add this amount of a normal serum (human or monkey) to the salt solution used as a diluent in the test.

We have found that 0.4 cc. of the serum is a satisfactory amount for the test. When this quantity is mixed with the virus suspension and injected, the test is so sensitive that sera from yellow fever convalescents diluted to 1 per cent usually protect a considerable proportion of the mice.

Technic of the Test

Specimens to be Examined.—At least 6 cc. of the serum to be tested is needed. The test requires 3 cc. The remainder is used in case the results of the first test are doubtful or the control tests prove unsatisfactory.

The Mice.—Healthy young adult white mice of about 20 gm. weight are preferred, but moderate differences in age do not seem to affect the results of the tests. A pure strain of mice, tested and found highly susceptible and bred on the premises, would give the best results. Satisfactory work may be done with susceptible mice purchased from dealers, but each supply from a new source should be tested for susceptibility and there should be an understanding with the dealer that he will not change the source of his mice without notifying the laboratory. During the test mice may be fed exclusively on bread soaked in milk, given once each day, and they will then require no water. During the experiments the mice are kept in glass battery jars with wire mesh covers, six mice in each jar. Wood shavings are placed in the jars, and the mice are moved to fresh jars 1 week after inoculation.

Preparation of the Virus.—An approximate time is set in advance for the tests, preferably 2 or 3 consecutive days in the middle of the week. 5 days before each testing date, a sufficient number of healthy mice are inoculated intracerebrally with yellow fever virus in mouse brain tissue. The mice are inspected each morning. The definitely sick animals are killed with chloroform and are pinned out, back down. A rapid and simple necropsy is performed to find out if there are gross

lesions of any disease other than yellow fever and to drain the blood from the brain. The only gross lesion probably due to yellow fever, which has been observed, is hemorrhage into the stomach and intestines, and this is absent in most cases. The mouse is then turned over and pinned out. The skin is slit with scissors from the nose to the middle of the back and the flaps are laid back. An assistant sears the top of the skull with a red-hot soldering iron. The operator removes the bony covering over the brain with sterile sharp-pointed scissors and then spoons out the brain with a rigid, narrow spatula of nickel. The brain is placed in a small, weighed petri dish. The other brains secured are placed in the same dish and the total weight is ascertained. The brains are then finely ground up in a porcelain mortar with enough isotonic salt solution, or preferably salt solution containing 10 per cent of normal serum, to make a 10 per cent suspension.

Preparation of the Serum-Virus Mixtures.—In advance of the tests, 3 cc. of each specimen of serum or diluted serum and of each of several sera for the controls is placed in a small test-tube. If less than 3 cc. of serum is available for a test, a 50 per cent solution of the serum may be tested if the dilution is stated in the report. If less than 1.5 cc. is received, we discard the specimen unless its unusual importance justifies testing it in higher dilutions. To each tube is added 1.5 cc. of the virus suspension. The contents of each tube are mixed and drawn up into a 5 cc. graduated glass syringe with the number of the specimen or that of the mouse group to be inoculated written on it with a wax pencil.

The Starch Solution.—The starch solution for intracerebral injection is prepared in advance by adding 2 per cent of corn-starch, such as is sold for food, to the 0.9 per cent sodium chloride solution and heating in a flask in a bath of boiling water. The solution is then placed in small wide mouthed Erlenmeyer flasks, autoclaved, and stored ready for use. In the morning of the day of the tests the starch solution is drawn up into tuberculin syringes of 0.25 cc. capacity, fitted with hypodermic needles 0.42 mm. in diameter (Stubs gauge No. 27) and 10 mm. long. The number of syringes filled is the same as the number of mouse groups to be inoculated.

Controls.—With each set of tests there should be five control groups of six mice each. Two groups should receive 0.4 cc. of normal serum (human or monkey) in place of the unknown serum of the test, and two should receive a known immune serum (human or monkey). To conserve the immune serum, it may be diluted to 10 per cent if of sufficiently high titer. The fifth control should be given the virus mixture intracerebrally. It shows whether the mice are susceptible and the virus potent. Intracerebral inoculation brings the animals down 1 day sooner than intraperitoneal inoculation with simultaneous cerebral injury.

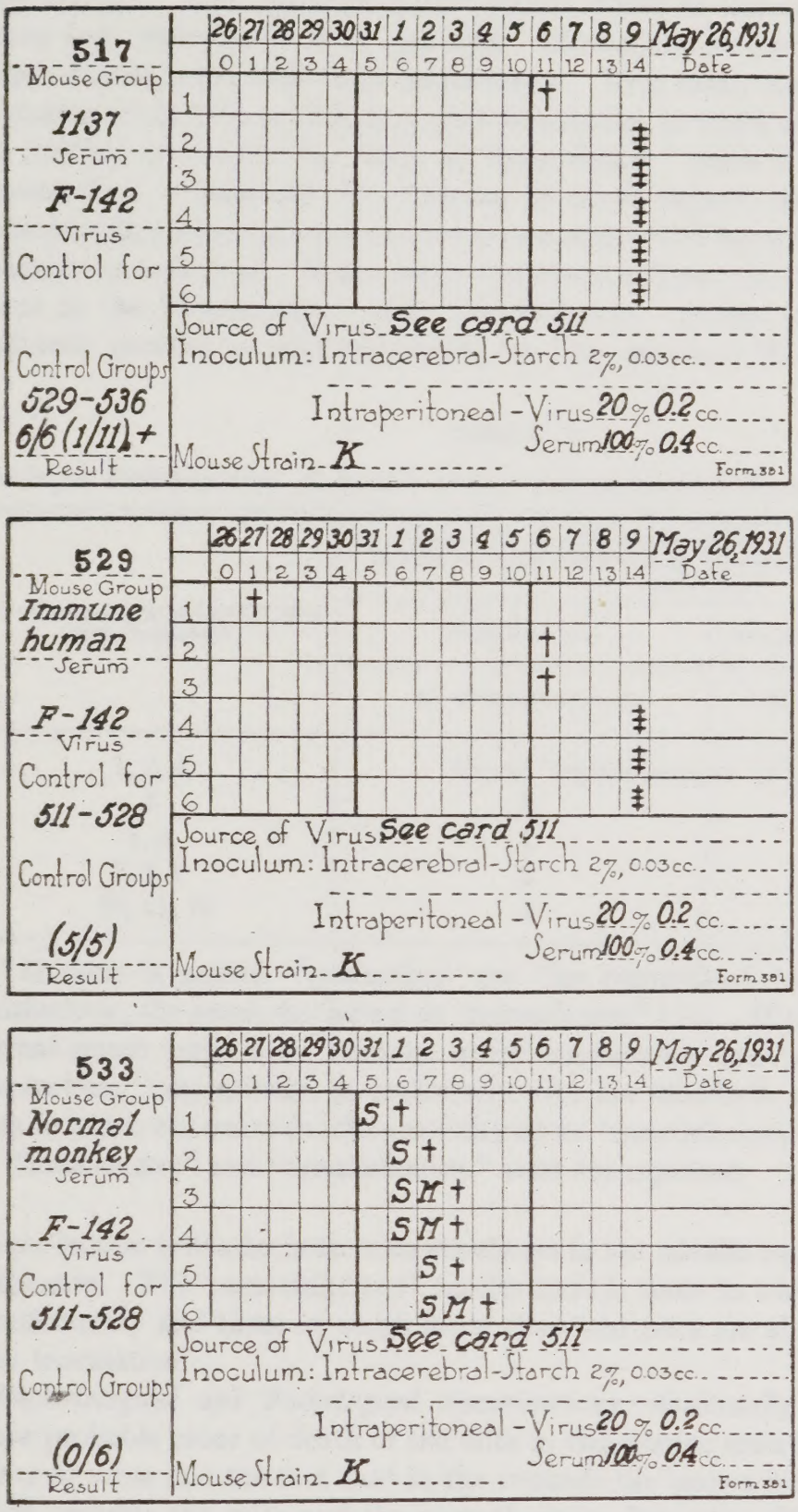
An important object of the immune serum control is to show that the deaths caused by the virus suspension injected are in reality due to yellow fever and not to some contaminating organisms in the virus mixture. In our recent experience the most frequent cause of an unsatisfactory result requiring repetition of the tests is contamination of the virus as revealed by deaths of mice in the immune serum control groups. The organism of mouse typhoid, Strain 1, was isolated from the brain of one of the animals dying in an unsatisfactory test of this kind. We are

attempting to diminish this difficulty by using healthy mice of our pure strain bred on the premises for source of virus and inoculating them from mixtures containing the smallest possible number of brains. When it seems advisable as an additional precaution, we filter the brain suspension used in inoculating the source animals through Berkefeld N filters. It would be possible also to go back to stored, dried specimens of the virus which were obtained when it was apparently free from contamination. Before filtering the virus, the brain suspension should be centrifuged and the sediment discarded. The mouse brain virus may be easily preserved by preparing a suspension of the brain tissue in normal human or monkey serum, placing it in 1 cc. amounts in test-tubes, drying it in the frozen state, and then sealing the tubes and storing in the refrigerator. We have already preserved the virus in this way for several months. The essentials of the method have been published by Sawyer, Lloyd, and Kitchen (17).

Inoculation of the Mice.—An assistant anesthetizes the mice by placing several in a battery jar, on the bottom of which is a layer of cotton moistened with ether. The mice are taken from the jar when they become unconscious and are laid on the table near the operator. The operator, wearing rubber gloves, takes a mouse, lays it back up on a towel on the table, parts the hair of the head with a swab wet with 70 per cent alcohol and thrusts the fine needle of the tuberculin syringe through the thin skull and into the center of the brain. He injects 0.03 cc. of the starch solution. The mouse is then picked up by the skin of the neck, and its tail is held by the third finger of the same hand. With one of the larger syringes an intraperitoneal injection of 0.6 cc. of the virus-serum mixture is given. The mouse is then dropped into a numbered jar. One operator with an assistant can test up to 25 sera on each of 3 days of the week if he has an abundance of equipment ready in advance. The actual injections require approximately 3 minutes for each mouse group.

Inspection and Recording.—The mice are inspected every morning for 14 days, and a record is made of those that are sick or dead. Our printed record form on cards measuring 77 by 128 mm. is shown in Fig. 1. The first mouse to become sick or die becomes Mouse 1 of the group, and the second, No. 2, and so forth.

Interpretation of the Results.—The result of the protection test is recorded as the ratio of the number of mice surviving on the 10th day after inoculation to those that were alive and well on the 4th day, and this is followed in parenthesis by the corresponding ratio for the control groups receiving normal serum with virus, thus: "5/6 (0/12)," meaning that 5 out of 6 mice survived in the test group and none of 12 in the normal serum control groups. Deaths before the 5th day are in all probability not due to the yellow fever virus. Unless a note is made to the effect that the control groups receiving immune serum and virus gave unsatisfactory results, it is to be assumed that the mice in these groups were protected. From the record cards the results are then classified as "protection," "no protection," "inconclusive," or "unsatisfactory," in accordance with the requirements of the guide given in Table IV. Unless a very few deaths in the "protection" groups and a similar number of survivals in the "no protection" groups were allowed, the



Key to symbols used in records

† = Died

‡ = Killed when sick

‡ = Killed when well

S = Sick

M = Moribund

F-142 = French strain of virus, 142nd passage in mice

6/6 (1/11) = 6 of 6 mice were protected in test; 1 of 11 survived in normal serum controls

\+ = Serum protected

± = Result inconclusive

− = Serum did not protect

U = Test unsatisfactory

Heavy vertical lines bound time zone within which deaths are considered significant

FIG. 1. Part of record of test of a serum by the intraperitoneal protection test in mice, to determine protective power against yellow fever. Upper card contains record of test; other two show that of half the immune and normal serum controls.

"inconclusive" and "unsatisfactory" results would be excessively numerous and many tests would have to be repeated. Our experience leads us to consider the requirements in the table to be conservative. Very rarely there may be a number of deaths on the 11th or 12th days after inoculation, in which case it may sometimes be justified to classify the result as "inconclusive" when it would otherwise be "protection." Ordinarily the deferring of death beyond the 10th day shows a considerable protective action of the serum and would have almost the same significance as a survival. If the amount of serum will permit, the tests of the specimens in the "inconclusive" and "unsatisfactory" groups are repeated, and if definitely positive or negative results are then obtained they are accepted, but

TABLE IV

Guide for Interpretation of Results of the "Intraperitoneal Protection Test" in Mice

No. of mice living and well 4 days after inoculation	No. of deaths or survivals allowed among these mice 5 to 10 days after inoculation if the report is to be "protection" or "no protection," respectively*	
	Protection (+)	No protection or negative (−)
	No. of deaths allowed	No. of survivals allowed
1, 2, 3	Result "unsatisfactory" (U) in any case	
4	0	0
5, 6	1	1
7, 8, 9	2	2
10, 11, 12	3	3

* If there is neither "protection" nor "no protection" but the controls are satisfactory, the result is classed as "inconclusive" (±). If the results with the normal serum and known immune serum controls are not "no protection" and "protection," respectively, in conformity with the standards of the table, all the tests to which the controls relate are classed as "unsatisfactory" (U). If possible, all "inconclusive" and "unsatisfactory" tests are repeated.

the protection ratios for both tests are shown in the tabular reports to the senders of the sera. The "unsatisfactory" results include those in which the controls are unsatisfactory and those in which fewer than four mice are alive and well 4 days after inoculation.

Bacteriological and Pathological Examinations.—Ordinarily the identification of the probable cause of death of the mice in the routine tests rests on the known source of virus and the fact that in the controls the known immune yellow fever serum protects the mice and the normal serum does not. If there are circumstances suggesting that deaths may be due to contamination of the individual specimen of serum, cultures may be taken of the serum and histological examinations may be made of the brains of the mice. Such difficulties are rarely encount-

ered if the sera are collected with great care and in sufficient quantity. The test is hardly worth while if a shipment of sera contains many specimens that are contaminated or below 3 cc. in amount.

Demonstration of the Presence of Virus in the Blood of the Mouse

The intraperitoneal protection test in mice was devised on the supposition that yellow fever virus injected into the peritoneal cavity quickly found its way into the blood stream and was carried through the blood vessels of the brain. Theiler had shown that the blood contained little or no virus during the course of the disease, and we were unable to find virus in the blood after symptoms had appeared.

To demonstrate the virus in the circulating blood after inoculation and its absence during the course of the yellow fever encephalitis, we carried out the experiments recorded in Table V.

In the principal experiment, mice were each given intraperitoneal injections of 0.2 cc. of a 20 per cent suspension of brain virus, French strain, 124th passage in mice, together with 0.4 cc. of normal human serum. The amounts of virus and serum were the same as were then being used in our routine protection tests. The mice were divided into two series, A and B. One group of mice of Series A was bled from the heart under ether anesthesia 24 hours after the injection. Other groups were similarly bled 48, 72, 96, and 120 hours after inoculation. The blood of each group was heparinized and mixed and then injected intracerebrally in amounts of 0.03 cc. into groups of normal mice as a test for the presence of virus in the injected blood. The results are shown in Table V. A considerable but irregular proportion of the mice inoculated died within the usual time period for deaths from experimental yellow fever after intracerebral inoculation of fixed virus (4 to 9 days after inoculation).

The mice of Series B were bled in the same way, but the fresh untreated blood of each individual mouse was injected immediately into a group of normal mice. As before, the injections were intracerebral and consisted of 0.03 cc. of blood. The times of bleeding were the same except that the bleedings after 120 hours were omitted. The results were similar to those of Series A.

In the first supplementary test recorded in Table V, mice were inoculated as in the principal test. The virus was of the French strain, 108th passage in mice, and 0.2 cc. of a 20 per cent suspension was injected intraperitoneally into each mouse. The mice were bled 24 hours later and the blood was pooled and allowed to clot. The serum was injected into normal mice intracerebrally in amounts of 0.03 cc. The result was almost identical with that obtained in Series A of the principal experiment with the inoculation of pooled whole blood after the same time interval. Of eleven mice inoculated with the serum and surviving on the third day, nine succumbed.

In the second supplementary test, the mice were inoculated intraperitoneally with the same amount of virus, French strain, 109th and 110th passages in mice, mixed with normal human serum, but they were given also an intracerebral

TABLE V

Recovery of Yellow Fever Virus from the Circulating Blood of Mice at Varying Intervals after Massive Intraperitoneal Inoculation

	Hours between inoculation and bleeding of source animal					
	24 (1 day)	48 (2 days)	72 (3 days)	96 (4 days)	120 (5 days)	144 (6 days)
Principal Experiment, Series A						
No. of mice bled as source (blood heparinized and pooled)	5	5	4	4	2	
No. of mice inoculated intracerebrally with the mixed blood and alive 3 days later	11	10	10	10	10	
No. of these mice dying or moribund 4 to 9 days after inoculation	8	3	1	6	7	
Principal Experiment, Series B						
No. of mice bled as source (untreated blood of each used separately)	5	5	5	4		
No. of mice inoculated in groups with these separate bloods and alive 3 days later	25	22	24	19		
No. of these mice dying or moribund 4 to 9 days after inoculation	8	9	13	8		
Supplementary Experiments						
No. of mice bled as source (blood pooled)	6*					4†
No. of mice inoculated with the mixed serum and alive 3 days later	11					16
No. of these mice dying or moribund 4 to 9 days after inoculation	9					0

* First supplementary experiment.

† Second supplementary experiment.

injection of 0.03 cc. of 3 per cent starch solution to localize the virus and permit infection to take place. In this way it was hoped to find out whether the virus would disappear by the 6th day after injection, and, if it did, to ascertain whether it would reappear with the appearance of symptoms. Four of the mice which were

moribund or definitely ill 6 days after inoculation were anesthetized and bled from the heart. The mixed serum was injected intracerebrally, in the amount used in the previous experiments, into sixteen normal mice. All of these mice remained well throughout the observation period of 14 days. The four mice whose blood had been taken for injection were killed and their brains were ground up together to make a 20 per cent suspension. Twelve mice were inoculated intraperitoneally each with 0.2 cc. of this suspension mixed with 0.4 cc. of normal human serum. Twelve other mice were similarly inoculated, but a known human immune serum was substituted for the normal serum. All the mice were given also 0.03 cc. of 3 per cent starch solution intracerebrally. Of the mice receiving normal serum, one died early, and the remaining eleven died from 5 to 8 days after inoculation. Of the twelve receiving immune serum, all but one remained well for the entire observation period of 14 days. Thus was shown the presence of the virus in the brain and its identity as yellow fever virus. Evidently the virus had disappeared from the blood before the 6th day after inoculation and had not reappeared with the onset of the encephalitis and the production of much yellow fever virus in the brain tissues.

While the evidence from these several experiments strongly suggests that yellow fever virus was present in the circulating blood of mice every day from the 1st to the 5th after intraperitoneal inoculation and caused all, or most of, the deaths in the mice inoculated with the blood specimens, the presence of the virus has been confirmed by histological examinations and subinoculations only for specimens of blood taken 24 hours, 48 hours, and 96 hours after inoculation. The evidence follows.

Serum Taken after 24 Hours.—(First supplementary experiment, Table V.) One of the mice which had been inoculated with the serum was killed when moribund, and part of its brain was examined histologically. There were definite lesions of encephalitis, typical of those seen in yellow fever in the mouse. Half of the same brain was ground up with half the brain of another mouse in the same group, and normal mice were inoculated intracerebrally with the suspension. Of twelve mice alive on the 3rd day after inoculation, eleven died from 4 to 9 days after inoculation, and the brain of one was examined and found to show pronounced and typical lesions of yellow fever encephalitis. Further subinoculations were made similarly from this group, and of seventeen mice alive on the 3rd day, fifteen died from 4 to 9 days after inoculation. The brains of two other mice of the group receiving the 24 hour serum were ground up together and used in inoculating eighteen mice intracerebrally. All of them died 4 to 6 days later. The brains of two were examined and found to show lesions of encephalitis.

Blood Taken after 48 Hours.—(Principal experiment, Series B, Table V.) Brain tissue of one of the mice which had received 48 hour blood was injected intracere-

brally into normal mice. Of ten alive on the 3rd day, all died or were moribund 5 to 6 days after inoculation. The brains of two were examined histologically and found to present lesions of encephalitis consistent with those of yellow fever.

Blood Taken after 96 Hours.—(Principal experiment, Series B, Table V.) One of the mice which had been inoculated with 96 hour blood was killed when moribund, and part of its brain was examined histologically. The lesions of encephalitis were present and considered to be those of yellow fever. The remainder of this brain was ground up with the brain of another mouse in the same group, and twelve mice were inoculated intracerebrally with the suspension. All died or were moribund 5 days later. The brain of one was examined and found to present the picture of yellow fever encephalitis.

From these observations it seems that the mouse is unique, among the animals so far investigated, with regard to the circulation of yellow fever virus in its blood, inasmuch as the virus in the blood disappears before symptoms begin and does not reappear with the onset of obvious disease. After massive intraperitoneal inoculation the virus appears promptly in the blood and remains for several days. If the virus in the circulating blood is given an opportunity to attack the brain through a local injury and produces disease on the 6th day after inoculation, the virus is absent from the blood at the time the symptoms begin, although it is abundant in the brain tissue. It would seem that the mouse, like many other animals, permits the injected virus to circulate for a time regardless of whether a manifest infection is to take place, and that the virus disappears from the blood after a few days. If the encephalitis of yellow fever occurs later, the virus does not reappear in the blood. We do not yet know whether protective substances against yellow fever are produced in the mouse by intraperitoneal inoculation in the absence of the production of yellow fever encephalitis, but protective substances have been shown to appear in several other resistant warm blooded animals after such inoculation (13). There is a possibility that the rise of antibodies in the blood frees the blood of virus while permitting virus to continue its development or to persist in the central nervous system, just as yellow fever virus might remain for a time in the liver of an infected monkey after it had disappeared from the circulation with the rise of antibodies. We do not know whether the virus in the circulating blood increases in amount or is simply the virus introduced by massive inoculation. Whatever the process is that permits virus to be recov-

ered from the blood, it seems evident that it does not depend on the production of the encephalitis which characterizes experimental yellow fever in the mouse.

Differences in the Susceptibility of Strains of Mice to Yellow Fever Virus

In the first months of our study, while using the strain of white mouse bred at The Rockefeller Institute for general purposes, we were disappointed in the seeming low virulence of the virus strains used. Even the fixed French strain, which had become so highly virulent in the hands of Theiler (11), allowed many of the mice to survive after intracerebral inoculation. We found the mortality too low at that time to permit the development of a satisfactory protection test in mice. In October, 1930, there was an abrupt rise in the mortality to approximately 100 per cent, and we observed that this coincided with the purchase of mice from Dealer K at a time of shortage of the Institute stock. We later secured strains from other sources and tested their susceptibility. Through the kindness of Dr. Carrel we received mice of the Dilute Brown strain and of the albino Swiss strain. The latter strain had been brought to the Institute by Dr. Clara Lynch in 1926 from the laboratory of Dr. de Coulon of Lausanne, Switzerland. Mice were secured also from three dealers, K, M, and D.

The results of comparative tests for susceptibility are shown in Table VI, and may be briefly summarized. Of the three pure strains, the Swiss and the Dilute Brown were found to be highly susceptible and suitable for use in the protection test. The mortality was nearly 100 per cent for both strains, but the incubation period was slightly shorter in the Dilute Brown mice than in the Swiss.

The Institute stock strain was remarkably resistant. This resistance was manifest even in suckling mice. Unlike adult mice, very young mice will ordinarily contract yellow fever encephalitis when inoculated by intraperitoneal injection in the absence of cerebral injury, as has been shown by Theiler (11). Suckling mice of the Institute stock, however, were found to be highly resistant to intraperitoneal inoculation, while similar mice from Dealer K were very susceptible.

This experience brings out the importance of using a pure strain of mouse of known susceptibility in yellow fever studies and protection

TABLE VI

Comparative Tests of Strains of Mice for Susceptibility to Yellow Fever Virus of the French Strain after 109 to 138 Passages in Mice

Comparative tests		Pure strains			Mixed strains		
		Swiss	Dilute Brown	Institute stock	Dealer K	Dealer M	Dealer D
*Intracerebral Inoculation of Young Adult Mice**							
Test 1	No. of mice			55		52	
	Percentage dead or moribund 4 to 9 days after inoculation			65%		96%	
Test 2	No. of mice			48	95		
	Percentage dead or moribund			50%	98%		
Test 3	No. of mice				53		54
	Percentage dead or moribund				100%		81%
Test 4	No. of mice	30	30		30		
	Percentage dead or moribund	100%	100%		83%		
Test 5	No. of mice	23	24		23	22	
	Percentage dead or moribund	100%	100%		100%	100%	
	No. dead or moribund on 4th day after inoculation	3	17		7	2	
	No. dead or moribund on 5th day after inoculation	19	7		16	20	
Intraperitoneal Inoculation of Suckling Mice 13 to 16 Days Old†							
Test 6	No. of mice			13	18		
	Percentage dead or moribund 5 to 10 days after inoculation			0%	83%		
	No. dying 11 to 15 days after inoculation			4	1		

* Inoculum: 10 per cent suspension of brain of moribund mouse in isotonic sodium chloride solution. Amount injected intracerebrally: 0.03 cc.

† Same inoculum as used intracerebrally. Amount injected intraperitoneally into suckling mice: 0.1 cc.

tests. Unfortunately, this is not always possible, and we are still compelled to buy reasonably susceptible mice of unknown and mixed heredity from dealers, while an insufficient but increasing supply of mice of the Swiss strain is being bred for us.

Titration of Sera for Protective Power

The technic of the intraperitoneal protection test in mice is well adapted to the titration of sera for their protective power against yellow fever. It is merely necessary to substitute serial dilutions of the serum for the full strength serum in the test. The methods of recording and interpreting remain the same.

We may take as an example the results of a titration in a study of the persistence of yellow fever immunity (9). The serum was from a woman who had had yellow fever 78 years before. The upper card in Fig. 1 shows the record of the full strength serum in this titration. The controls were satisfactory; the normal serum controls permitted only one of eleven mice to survive. The protection ratios and results for the several dilutions were: dilution 1:1, 6/6 (+) meaning that six mice out of six survived; 1:2, 6/6 (+); 1:4, 6/6 (+); 1:8, 4/5 (+); 1:16, 4/6 (±); 1:32, 4/6 (±); 1:64, 1/6 (−); 1:128, 1/6 (−). By the rigid standard of Table IV, the specimen of serum protected in dilutions up to 1:8, and gave inconclusive results at 1:16 and 1:32, and gave no protection in higher dilutions. The titer would be 1:8.

Sensitivity of the Test

The intraperitoneal protection test in mice is much more sensitive than the protection test as made in monkeys. Sera which protect monkeys also protect mice, but many which protect mice do not protect monkeys.

The serum which was mentioned above as protecting mice in a dilution of 1:8 was tested also in two monkeys. One was given 3 cc. of the serum per kilo of body weight and the other 1.5 cc. per kilo by intraperitoneal injection, and each received a subcutaneous injection of 0.4 cc. of Asibi yellow fever virus in monkey blood 6 hours later. All of three control animals died of yellow fever. The test animals both developed fever and recovered, showing that they had received definite protection but of a low degree. The sera of several persons who have recovered in recent years from laboratory infections usually protect monkeys completely when as much as 1 cc. per kilo is injected and protect mice when the dilution of the serum is 1:50 or 1:100. The intraperitoneal test in mice has been found sufficiently sensitive to reveal active immunity in persons and monkeys successfully protected

against yellow fever by means of inoculation with mixtures of immune serum and mouse brain virus.

Danger to Personnel

Unusual precautions are necessary to prevent the infection of laboratory personnel with yellow fever virus from infected mice. Three such infections are known to have occurred. We have considered it necessary to limit the personnel working in the rooms in which the mice are inoculated and the brains removed, to persons known to have become immune to yellow fever as the result of an attack of the disease or through vaccination.

SUMMARY AND CONCLUSIONS

1. A method of testing sera for protective power against yellow fever is described and designated as the intraperitoneal protection test in mice.

2. The test consists essentially of the inoculation of mice intraperitoneally with yellow fever virus, fixed for mice, together with the serum to be tested, and the simultaneous injection of starch solution into the brain to localize the virus. If the serum lacks protective power the mice die of yellow fever encephalitis.

3. The test is highly sensitive. Consequently it is useful in epidemiological studies to determine whether individuals have ever had yellow fever and in tests to find whether vaccinated persons or animals have in reality been immunized.

4. When mice were given large intraperitoneal injections of yellow fever virus fixed for mice, the virus could be recovered from the blood for 4 days although encephalitis did not occur. If the brain was mildly injured at the time of the intraperitoneal injection, the symptoms of yellow fever encephalitis appeared 6 days later, but the virus was then absent from the blood.

5. Strains of white mice vary greatly in their susceptibility to yellow fever.

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